NUCLEIC SEQUENCES ENCODING AN AT2 RECEPTOR-INTERACTING PROTEIN (ATIP) AND THEIR APPLICATIONS

The present invention relates to nucleic sequences encoding a protein capable of interacting with the AT2 receptor, to oligonucleotides contained in the said sequences, to their applications as probes and for the expression of the said proteins, to the vectors useful for the said expression, to the host cells containing the said vectors and to a model for studying the AT2 receptor.

The present invention also relates to the said proteins and to their applications.

The octapeptide, angiotensin II, mainly known as a regulator of blood pressure, has also been described as an important modulator of cell growth. Interestingly, this peptide appears to exert opposite effects on cell growth according to whether it is bound to one or the other of its two subtypes of membrane receptors (AT1 or AT2).

The AT2 receptor subtype, which also belongs to the G protein-coupled receptor family, is still poorly characterized both from the point of view of its mechanisms of activation and its physiological role (C. Nahmias et al., *Trends Pharmacol Sci*, 1995, 16, 223-225). Several arguments suggest, however, a role for this receptor in the phenomena of cell proliferation, differentiation or adhesion.

The AT2 receptor is highly expressed during foetal life, disappears in adults in most tissues, but becomes reexpressed under pathophysiological conditions involving restructuring of the tissues.

Studies carried out *in vivo* have demonstrated the inhibitory role exerted by the AT2 subtype on the proliferation of the muscle cells of the *tunica intima vasorum* after vascular lesion (P. Janiak et al., *Hypertension*, 1992, 20, 737-745; M Nakajima et al., *Proc. Natl. Acad. Sci.* USA, 1995, 92, 10663-10667).

Moreover, the stimulation of the AT2 receptor activates phosphatase SHP-1 (Bedecs K., et al; *Biochem. J.*, 1997, 325, 449-454). The fact that the AT2 receptor activates a phosphatase is consistent with its antiproliferative effects.

In the light of the above, it has been shown that, on cells in culture, the AT2 receptor:

- inhibits the synthesis of DNA and proliferation, which are induced by angiotensin II (Ang II) and bFGF (M. Stoll et al., *J. Clin. Invest.*, 1995, 95, 651-657),
- induces apoptosis (T. Yamada et al., *Proc. Natl. Acad. Sci. USA*, 1996, 93, 156-160), and
- induces neuronal differentiation (L. Laflamme et al., *J. Biol. Chem.*, 1996, 271, 22729-22735).

Studies of the signalling pathways associated with the AT2 receptor have been undertaken in cells of the N1E-115 line which are derived from a murine neuroblastoma and which express only the AT2 subtype. A first study has made it possible to demonstrate rapid and transient dephosphorylation of some proteins on the tyrosine residues following the treatment of N1E-115 cells with angiotensin II (C. Nahmias et al., *Biochem. J.*, 1995, 306, 87-92). It has also been shown that the AT2 receptor interferes with the pathways for activation of growth factor receptors and inhibits the activity of MAP kinases (ERK1 and ERK2) *(mitogen-activated protein)*, which play a key role in the phenomena of cell proliferation and differentiation. The inhibitory effect of AT2 on the activation of MAP kinases is rapid and transient, does not involve a regulatory protein sensitive to the pertussis toxin (of the Gi/Go type), but involves the activation of an orthovanadate-sensitive tyrosine phosphatase.

Taking into account the role of the AT2 receptor in cell proliferation, the inventors have sought to develop tools capable of regulating the action of the AT2 receptor. Indeed, the activation of

the AT2 receptor may have repercussions in cancerology (inhibition of cell proliferation).

In general, the AT2 receptor has opposite effects to those of AT1 on the activation of MAP kinases and on cell proliferation; study of the communication which may exist between these two receptor subtypes, which bind the same ligand, is consequently of interest.

The study of the signalling pathways and of the regulation of the AT2 receptor also represents a major stake for human health knowing that antagonists of the AT1 receptor are currently administered to patients with hypertension. In this context, it is essential to know the biological effects associated with the AT2 receptor which remains activable by circulating Ang II in this type of treatment.

The subject of the present invention is an isolated nucleic acid (DNA or RNA) fragment, encoding a protein capable of binding to the AT2 receptor, which fragment is selected from the group consisting of the sequences SEQ ID NO:1, 3, 5, 7 and 9, as represented in the sequence listing included in the present application.

These various sequences correspond to the complementary DNA (cDNA) encoding all or part of the protein called hereinafter ATIP (*AT2 interacting protein*).

The sequence SEQ ID NO:1 (1803 bp) corresponds to the complete nucleic sequence of mouse ATIP and includes both the parts encoding the AT2 receptor binding protein and the noncoding parts.

The sequence NO:3 (1323 bp) corresponds to the nucleic acid sequence of the coding part of the sequence SEQ ID NO:1, while the sequence SEQ ID NO:5 corresponds to the sequence NO:1 fragment obtained by the two-hybrid technique (A Plessis et al., M/S, 1994, 9, I-1K; J. Luban et al., *Curr. Op. Biotechnol.*, 1995, 6, 59-64).

The sequence SEQ ID NO:7 (3742 bp) corresponds to the complete nucleic sequence of the human cDNA and includes both the parts encoding the protein homologous to the mouse ATIP and the noncoding parts.

The sequence SEQ ID NO:9 (1308 bp) corresponds to the coding part of the sequence SEQ ID NO:7.

The subject of the present invention is also transcripts, characterized in that they are complementary to the sequences in accordance with the invention and are in particular generated from the said sequences.

The subject of the present invention is, in addition, fragments of the said sequences comprising between 20 and 400 bp, useful as probes or as primers, for the detection of the sequences SEQ ID NO:1, 3, 5, 7 or 9, or of homologous sequences.

Among the said fragments, there may be mentioned in particular a probe of 354 bp (SEQ ID NO:5) as well as any fragment of 20 bp to 400 bp included in the sequences SEQ ID NO:1, 3, 5, 7 or 9.

As primer, there will be used in particular the sequence SEQ ID NO:10 (antisense oligonucleotide) which makes it possible in particular to amplify the 5' parts of the various mRNAs corresponding to ATIP (5' RACE technique: Marathon cDNA amplification kit, Clontech).

It is also possible to use, as amplification primers, any pair of oligonucleotides of more than 20 bp and comprising part of the ATIP (human or mouse) nucleic sequence, in particular the pair SEQ ID NO:11-SEQ ID NO:12.

The preferred hybridization (prehybridization and hybridization) conditions are in particular the following: 45% formamide, 9% dextran sulphate, 0.2% BSA, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 0.01% SDS, 0.05 mM Tris pH 7.5, 0.9 M NaCl and rinses to a stringency corresponding to the buffer: 1XSSC, 0.1% SDS.

The subject of the present invention is also a purified and isolated protein, called ATIP, which is capable of interacting with the AT2 receptor and which is selected from the group consisting of the sequences SEQ ID NO:2, 4, 6 or 8.

The murine and human sequences exhibit 85.6% homologies. The human sequence (human ATIP) possesses 5 amino acids less than the mouse sequence (mouse ATIP). The amino acids missing from the human sequence are situated at the level of amino acids: 162, 163, 164, 166 and 214 of the mouse ATIP sequence.

Comparisons (Blast) between the ATIP protein sequences according to the invention and the sequences contained in data banks indicate that human ATIP (like mouse ATIP) never exhibits more than 25% homology with a known sequence, and this being the case only over part of this sequence.

The subject of the present invention is also a translational product, characterized in that it is encoded by a nucleotide sequence in accordance with the invention.

The subject of the present invention is, in addition, antibodies, characterized in that they are directed against the ATIP protein or an ATIP protein fragment according to the invention.

The subject of the present invention is also a recombinant cloning and/or expression vector, characterized in that it comprises a nucleotide sequence in accordance with the invention.

The subject of the present invention is also a transformed host cell, characterized in that it comprises a vector as defined above.

Among the preferred transformed cells according to the invention, there may be mentioned *E. coli* and CHO cells.

The subject of the present invention is also transformed host cells, characterized in that they consist of a suitable yeast strain cotransformed with

at least two vectors which respectively encode (i) a so-called bait protein selected from the group consisting of a fragment containing at least SEQ ID NO:5 of the ATIP protein and a fragment containing at least the C-terminal end of the AT2 receptor, which bait protein is fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the same transcription factor and (ii) a so-called prey protein, selected from the group consisting of a fragment containing at least SEQ ID NO:5 of the ATIP protein, a fragment containing at least the C-terminal end of the AT2 receptor and any other polypeptide corresponding to a sequence contained in a cDNA library, which prey protein is fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the same transcription factor, which vectors comprise, in addition, selectable markers.

According to an advantageous embodiment of the said cells, they consist in particular of:

- either a suitable yeast strain cotransformed with three vectors which respectively encode (i) a bait corresponding to a fragment containing the C-terminal end of the AT2 receptor fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor, (ii) a fragment containing at least SEQ ID NO:5 of the ATIP protein according to the invention, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor and (iii) a polypeptide corresponding to a sequence contained in a cDNA library, which vectors comprise, in addition, selectable markers,

- or a suitable yeast strain cotransformed with two vectors which respectively encode (i) a fragment

containing at least SEQ ID NO:5 of the ATIP protein according to the invention, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor and (ii) a polypeptide corresponding to a sequence contained in a cDNA library, fused with a protein selected from the group consisting of the DNA-binding domain of the transcription factor and the activation domain of the said transcription factor, which vectors comprise, in addition, selectable markers,

- or a suitable yeast strain cotransformed with two vectors, namely (i) a vector encoding a fragment containing at least SEQ ID NO:5 of the ATIP protein, mutated or not, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor and (ii) a vector encoding a fragment containing the C-terminal end of the AT2 receptor, mutated or not, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor, which vectors comprise, in addition, selectable markers, one of the two vectors necessarily encoding a mutated protein.

The subject of the present invention is also a method for selecting proteins inhibiting ATIP protein according to the invention-AT2 receptor interaction, which method comprises:

(a) cotransforming a suitable yeast strain with three vectors which respectively encode (i) a bait corresponding to a fragment containing the C-terminal end of the AT2 receptor fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor, (ii) a fragment containing at least SEQ ID NO:5 of the ATIP protein according to the invention, fused with a protein selected from the

group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor and (iii) a polypeptide corresponding to a sequence contained in a cDNA library, which vectors comprise, in addition, selectable markers,

(b) selecting the clones of cDNA library expressing a polypeptide inhibiting the AT2 receptor-ATIP protein according to the invention interaction, on an appropriate selective medium, and

(c) identifying the said polypeptide.

Such a method uses in particular the so-called reverse two-hybrid or three-hybrid technique as described in Vidal et al. (*Proc. Natl. Acad. Sci.* USA, 1996, **93**, 10315-10320 and 10321-10326) or Tirode et al. (*J. Biol. Chem.*, 1997, **272**, 37, 22995-22999).

The subject of the present invention is also a method for screening polypeptides interacting with the ATIP protein according to the invention, which method comprises:

(a) cotransforming a suitable yeast strain with two vectors as defined above, namely which respectively encode (i) a fragment containing at least SEQ ID NO:5 of the ATIP protein according to the invention, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor and (ii) a polypeptide corresponding to a sequence contained in a cDNA library, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor, which vectors comprise, in addition, selectable markers, and

(b) selecting the clones expressing a polypeptide interacting with the ATIP protein, on a suitable selective medium.

Such a method makes it possible in particular to search for other proteins interacting with the ATIP

protein, in particular in order to find the next links in the pathway activated by the AT2 receptor, so as to use them to modify the protein according to the invention-AT2 receptor interaction.

The subject of the present invention is also a method for characterizing the domains involved in the ATIP protein-AT2 receptor interaction, characterized in that it comprises:

(a) cotransforming a suitable yeast strain with two vectors, as defined above, namely (i) a vector encoding a fragment containing at least SEQ ID NO:5 of the ATIP protein, mutated or not, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor and (ii) a vector encoding a fragment containing the C-terminal end of the AT2 receptor, mutated or not, fused with a protein selected from the group consisting of the DNA-binding domain of a transcription factor and the activation domain of the said transcription factor, which vectors comprise, in addition, selectable markers, one of the two vectors necessarily encoding a mutated protein, and

(b) visualizing, by selection on a suitable selective medium, the possible loss of the ATIP-AT2 receptor interaction.

Such a method makes it possible to identify and to delimit the important domains of the ATIP protein or of the C-terminal end of the AT2 receptor, on which their interaction depends, so as to use them as preferred target for modifying the AT2 receptor signalling.

The subject of the present invention is also a method for selecting substances capable of influencing the ATIP protein according to the invention-AT2 receptor interaction, which method comprises:

(a) bringing the ATIP protein, attached to a support, into contact with a fusion protein AT2

receptor-protein tag, optionally in the presence of a substance to be tested,

(b) at least one washing of the said support thus treated with a suitable buffer, and

(c) visualizing the possible ATIP-AT2 receptor interaction, in particular in SDS-PAGE, followed by immunoblotting with antibodies directed against the protein tag, fused with the AT2 receptor, or against the AT2 receptor.

If the substance to be tested inhibits the ATIP-AT2 receptor interaction, the visualization step is negative.

In accordance with the invention, ATIP is attached to the said support either covalently, or through affinity binding between an attachment substance fused with ATIP and the said support. For example, the said support consists of beads coupled either to a substance having affinity with the said attachment protein, fused with ATIP, or to suitable antibodies.

The fusion protein AT2 receptor-protein tag is in particular obtained from a lysate of cells transfected with a vector expressing the fusion protein AT2-protein tag.

As a variant, the said method for selecting substances capable of interacting with the ATIP protein according to the invention comprises:

(a) bringing the ATIP protein, attached to a support, into contact with a cell lysate,

(b) at least one washing of the said support thus treated with a suitable buffer,

(c) visualizing the possible protein combined with the ATIP protein, in particular in SDS-PAGE, followed by immunoblotting with appropriate antibodies, and

(d) identifying the protein in the cell lysate interacting with the ATIP protein.

In accordance with the said method for selecting substances capable of influencing the ATIP protein according to the invention-AT2 receptor interaction, it is possible to use in particular, as fusion proteins ATIP-protein tag, the proteins GST-ATIPc and MYC-ATIPc, which constitute tools which can make it possible to bring down *in vitro* any proteins interacting with ATIP, for example, from cell lysates activated or otherwise with ligands for the AT2 receptor. The GST-ATIP protein may be brought down by specific interaction of GST with agarose beads coupled to glutathione, or alternatively immunoprecipitated with the anti-ATIP antibody. The *Myc*-ATIP protein may be immunoprecipitated with commercial anti-MYC antibodies or with the anti-ATIP antibody.

The advantage of these methods consists in finding means of modifying the signalling, the level of expression or the pharmacology of the AT2 receptor, which may have therapeutic applications. Indeed, when a pathological condition has been clearly correlated with a transduction abnormality associated with the AT2 receptor, modification of this transduction, in particular by acting on the binding of the AT2 receptor to the protein according to the invention, may then possibly compensate for the pathological disorder or at least influence it.

The subject of the present invention is also the use of the abovementioned cotransformed cells for the selection and screening of substances or of proteins capable of influencing the ATIP protein-AT2 receptor interaction or capable of interacting with the ATIP protein.

In addition to the preceding features, the invention also comprises other features which will emerge from the description which follows, which refers to exemplary embodiments of the method which is the subject of the present invention as well as to the accompanying drawings, in which:

- Figure 1 corresponds to the C-terminal end of the mouse AT2 receptor, used as a two-hybrid bait for screening a mouse cDNA library;

- Figure 2 illustrates the position of the GAL4-binding domain and the multiple cloning site of the plasmid pGBT9 (Clontech);

- Figure 3 illustrates the presumed *coiled-coil* structures (*coiled-coil* domains underlined) of mouse ATIP;

- Figure 4 illustrates the presumed *coiled-coil* structures (*coiled-coil* domains underlined) of human ATIP;

- Figure 5 illustrates the structure of the plasmid pVP16;

- Figure 6 illustrates the multiple cloning site of the plasmid pRSET A;

- Figure 7 illustrates the MCY sequence used to construct the plasmid pcDNA3-MYC;

- Figure 8 illustrates the structure of the plasmid pBAC-PAK-poly HIS;

- Figure 9 illustrates a Northern blot of several human tissues hybridized with the probe ATIPmouse-short (SEQ ID NO:5);

- Figure 10 illustrates the interaction *in vitro* of the protein ATIPmouse-short with the C-terminal end of the AT2 receptor; and

- Figure 11 illustrates the modifications of the signal induced by the AT2 receptor by overexpression of the ATIP protein.

It should be clearly understood, however, that these examples are given solely by way of illustration of the subject of the invention and do not constitute in any manner a limitation thereto.

EXAMPLE 1: Demonstration of a specific protein-protein interaction between the AT2 receptor and the protein having the sequence SEQ ID NO:6 according to the invention

Materials and methods

- The two-hybrid system, initially developed by Song and Fields in 1989 (Nature, **340**, 245-246) is based on the fact that the activity of numerous eukaryotic transcription-activating factors requires only two domains: an activating domain which does not have the capacity to bind DNA and a DNA-binding domain.

In the two-hybrid system, the DNA-binding domain is fused with a protein X and the activation domain is fused with a protein Y. If, and only if, X and Y interact, a complex is formed which reconstitutes a functional transcription factor.

- Construction of the expression vectors:

. "bait" vectors:

Protein X: C-terminal end of the sequence encoding the mouse AT2 receptor (52 amino acids of CVNPF at the stop codon, see Figure 1), fused with the sequence encoding the Gal4 DNA-binding domain (Figure 2).

Insert: end of the mouse AT2 receptor (159 bp + 16 bp of sites generated by PCR) inserted at the level of the EcoRI and BamHI sites of the vectors pLEX9 (Clontech) or pGBT9 (modified pGAD424 or pBTM116; A.B Vojtek et al., Cell, 1993, **74**, 205-214).

The following sequence is thus obtained:
CGGAATTC on the 5' side-AT2 C-terminal sequence of 52 amino acids-GGATCCCG 3' side

. screened library:

mouse foetal cDNA library (A.B. Vojtek et al., *Cell*, 1993, **74**, 205-214), containing inserts of 350 to 700 bp (protein Y) in the vector VP16 (Figure 5).

. "Bait" control vectors

Protein X: C-terminal end of the human β2-adrenergic receptors, rat AT1 or human bradykinin.

. Transformed yeast strain

HF7c (Clontech) for the bait constructed in pGBT9;

L40 for the bait constructed in pLex9.

Results

This strategy made it possible to isolate a clone derived from the cDNA library containing an insert of 354 bp (ATIP) which interacts specifically with the C-terminal end of AT2. It is of interest to note that the screening of this library with the constructs produced in the two expression vectors pGBT9 and pLEX9 made it possible to find this same clone in both cases. This clone does not interact with control proteins exhibiting nonspecific interactions.

To evaluate the selectivity of this interaction, the ATIP clone was tested as a two-hybrid system with the C-terminal ends of the receptors: human β2 adrenergic, rat AT1 and human bradykinin, and all gave negative results. This indicates that the polypeptide encoded by the ATIP clone interacts, in a selective manner, with the C-terminal end of the mouse AT2 receptor.

EXAMPLE 2: Characterization of the ATIP clone

To test for the corresponding whole clone, a probe of 354 bp (SEQ ID NO:5), which corresponds to the insert obtained by digestion with the restriction enzyme NotI of the plasmid isolated in a two-hybrid system (that extracted from the VP16 library, selected as being positive in the screen using, as bait, the C-terminal end of the mouse AT2 receptor), is used to screen a mouse foetal cDNA library constructed with inserts of more then 1 kb in size. Two overlapping clones, comprising the ATIP sequence, were thus identified and made it possible to sequence 1803 bp of the corresponding cDNA (SEQ ID NO:1). This sequence contains an open reading frame of 1323 bp (SEQ ID NO:3), potentially encoding a protein of 440 amino acids (SEQ ID NO:2 and 4). Comparisons between the

identified protein sequence and the sequences contained in data banks indicate that it never exhibits more than 25% homology with a known sequence part.

The 354 bp probe (SEQ ID NO:5) was used as probe in Southern and Northern in a very satisfactory manner under the hybridization conditions below: prehybridization and hybridization in 45% formamide, 9% dextran sulphate, 0.2% BSA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 0.01% SDS, 0.05 mM Tris pH 7.5, 0.9 M NaCl and rinses to stringency: 1 × SSC, 0.1% SDS.

In parallel, Northern blot hybridization experiments carried out on total RNAs of N1E-115 cells with the ATIP probe (SEQ ID NO:5) confirm the expression of the corresponding mRNA in the N1E-115 cells, and indicates the existence of at least 5 transcripts of different sizes. These transcripts correspond to alternative splicings of the same gene or to different homologous genes.

On a Northern, performed under the conditions described in the literature on a 5 µg sample of poly A+ RNA of N1E-115 cells, the sizes of the various transcripts hybridizing with the ATIPmouse probe are = 2.5-3.5-5-5.3 and 7.5 kb.

Figure 9 represents a Northern blot containing poly A+ RNAs of various human tissues, hybridized with the same ATIPmouse probe. It is possible to observe that ATIP is ubiquitously expressed. A predominant transcript at 4.4 kb is found in all the tissues represented, to which there are added, according to the tissues, other longer transcripts (pancreas and heart) or shorter transcripts (pancreas, skeletal muscle, placenta, brain and heart). These are perhaps the fruit of an alternative splicing of the ATIP RNA which would be dependent on the tissue considered or alternatively they are the sign of the existence of an RNA family encoding proteins of the "ATIP family" homologous to

ATIP and which are revealed by the probe, at the stringency used.

To know the size of the smallest transcript encoding ATIP, a rapid amplification of the cDNA ends (5' RACE, Marathon cDNA Amplification Kit from Clontech) from poly A+ RNA of N1E-115 cells was carried out using the antisense oligonucleotide of SEQ ID NO:10, to amplify the 5' parts of the various mRNAs corresponding to the endogenous ATIP of the N1E-115 cells (murine neuroblastoma).

The results obtained indicated that the smallest transcript including the ATIP domain is an mRNA of 1950 bp, which indeed contains the start of the coding sequence obtained by cloning.

Any other pair of oligonucleotides (primers) of more than 20 bp and comprising part of the ATIP sequence may also be used to amplify, by PCR (PCR conditions to be determined for each pair of oligonucleotides with the aid of the OLIGO 4 software), part of the ATIP (and to give a DNA fragment which may be optionally used as a probe to recognize the DNA or the RNA corresponding to the ATIP).

EXAMPLE 3 Construction of various vectors according to the invention

In general, the vectors containing ATIPmouse-short (with the exception of pRSETA-ATIPmouse-short) were obtained from an insert produced by PCR with the following two oligonucleotides (SEQ ID NO:11 and SEQ ID NO:12):

oligo. sense: 5' CGCGGATCCCAGACAGACCGGACGGAACTGGAG3'

oligo. antisense: 5'CCGGAATTCACTACAACCTTTCGTTTAAAGCATC 3',

using as template the vector VP16-ATIPmouse-short (Figure 5). For the sake of convenience, this vector is called $^{B}ATIPc^{stop,E}$. Indeed, digested with BamHI and EcoRI, it gives an insert corresponding to the sequence

1st strand: GATCC-SEQ ID NO:5 (minus CAT)-TAGTG
2nd strand: CCTAG----------CTTAAG
(STOP)

BamHI site EcoRI site

Other vectors may also be constructed; they comprise all or part of the ATIP protein and are the following:

-VP16-ATIPmouse-short (vector taken from the library screened in the two-hybrid system, comprises 354 bp (SEQ ID NO:5), inserted in NotI into VP16).

-pCDNA3-MYC-ATIPmouse-short (insert ^{B}ATIPc^{stop,E}, inserted in BamHI-EcoRI into pCDNA3-MYC (pcDNA3 from Invitrogen, modified by insertion of the MYC sequence, Figure 7); this plasmid may be used in stable or transient transfections. It makes it possible to express MYC-ATIPmouse-short in eukaryotic cells. The expression of this protein in eukaryotic cells after transfection of the corresponding plasmid has already been obtained and checked by immunoreaction with an anti-MYC and anti-ATIP antibody.

-pRSETA-HIS-ATIPmouse-short (insert ^{B}ATIPc^{stop,E}, inserted in BamHI-EcoRI into pRSETA, Invitrogen). This plasmid makes it possible to express the fusion protein HIS-ATIPmouse-short in bacterial cells and to purify it on a nickel column (see Figure 6 for the multiple cloning site).

-pBacPAK-polyHIS-ATIPmouse-short (insert ^{B}ATIPc^{stop,E}, inserted in BamHI-EcoRI into the vector pBacPAK-polyHIS (commercial pBacPAK, modified by insertion of a sequence containing a histidine tag and a site for cleavage with thrombin, Figure 8). This construct may be used to express the ATIPmouse-short protein, fused with a histidine tag, in insect cells (SF9 type). Indeed, as indicated, this vector contains a poly-histidine insert and can therefore encode the fusion protein. The latter, like the fusion protein cloned into pRSET, may be purified on a nickel column and may serve in the same type of techniques.

-pGEX-4T1-GST-ATIPmouse-short (insert amplified by the PCR identical to ^{B}ATIPc^{stop,E}, but with no STOP codon, which extends the ATIPmouse-short sequence by the few amino acids which follow: Phe-Glu-Phe-Pro-Gly-Arg-Leu-Glu-Arg-Pro-His-Arg-Asp obtained from the plasmid pGEX-4T-1 (Pharmacia). This plasmid makes it possible to express the protein GST-ATIPmouse-short in bacterial cells and to purify it on glutathione-agarose beads.

-pCDNAI-ATIPmouse clone1 (entire 5' sequenced from ATIP and ORF up to bp: 1205 starting from the beginning of the clone, inserted in BstxI into pCDNAI). This plasmid is derived from the cloning of the mouse foetal library with the probe SEQ ID NO:5. This plasmid can serve to produce, in bacteria, the 5' portion of the ATIPmouse DNA, so as to use it as a probe.

-pCDNAI-ATIPmouse clone2 (2nd half of the ORF of ATIP from bp: 616 and up to the end of the 3'sequenced (bp 1803), inserted in BstxI into pcDNAI, Invitrogen). This plasmid can serve to produce, in bacteria, the 3' portion of the ATIPmouse DNA, so as to use it as a probe.

-pCDNAI-ATIPmouse-long (clones 1 and 2 placed end to end, using the intermediate SapI site. This plasmid contains the entire ATIPmouse clone, inserted in BstxI into pCDNAI). This plasmid may be used in transient transfections in eukaryotic cells.

-pCDNA3-ATIPmouse-long (whole ATIPmouse from BamHI-XbaI of pCDNAI-ATIPmouse-long, and inserted into pcDNA3, Invitrogen, at these same sites). This plasmid may be used in stable or transient transfections in eukaryotic cells. It made it possible to translate *in vitro* (kit TNT T7 *coupled reticulocytes lysate systems,* Promega) the whole ATIP protein and to observe that its translational product has an apparent molecular weight on gel of 58 kDa. Added to this predominant product are two minor products of 30 and 15 kDa. According to the ATIP sequence, these could correspond to partial

products of translation *in vitro* starting with ATGs other than that at position 178 of SEQ ID NO:1.

EXAMPLE 4: Production of stable clones expressing the ATIPmouse-short or long protein

Stable clones expressing both the human AT2 receptor and ATIPmouse-short (SEQ ID NO:6) or ATIPmouse-long (SEQ ID NO:3) were obtained by transfection.

CHO cells, deficient in dihydrofolate reductase, are transfected with a plasmid containing the region encoding the human AT2 receptor (Bedecs et al., *Biochem. J.* 1997, **325**, 449-454).

The clone selected, CHO-hAT2, expressing 100 fmol of AT2 receptor/mg of protein, is cultured on an HAMF12 medium supplemented with 10% foetal calf serum and used between passages 10 and 30.

This clone was itself transfected with the plasmids pCDNA3-MYC-ATIPmouse-short or pCDNA3-ATIPmouse-long described in Example 3. The selection of the clones stably expressing the ATIP protein (short form or long form) was carried out in a selective medium containing 800 µg/ml of G418. The cell lysates, corresponding to the various selected clones, were subjected to SDS-PAGE followed by immunoblotting and this was incubated with the anti-ATIP polyclonal antibody. The results obtained indicate that various clones expressing various levels of ATIPmouse-short were able to be obtained.

EXAMPLE 5: Production of polyclonal antibodies directed against the SEQ ID NO:6 sequence

To progress in the characterization of this clone, the production of polyclonal antibodies directed against the ATIP domain was undertaken.

For that, a vector encoding a protein corresponding to this domain fused with six histidine residues was constructed.

The following sequence:

GGA TCC-SEQ NO:5-TAG-TGA-ATT

is inserted into the plasmid pRSETA, as defined above.

In this insert, SEQ ID NO:5 does not comprise the first CAT.

The plasmid obtained is expressed in the *E. coli* strain BL 21 (DE3) (F^- ompT$^-$ r_B^- m_B^-) containing the bacteriophage DE3 which carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, the start of the *lacZ* gene and the gene encoding T7 RNA polymerase. This fragment is introduced into the *int* gene.

In the presence of DE3, only the lacUV5 promoter, inducible by IPTG directs the transcription of T7 RNA polymerase.

The addition of 0.4 mM IPTG to a culture of BL21 (DE3) cells induces the production of T7 RNA polymerase which, in turn, causes the transcription of the target DNA of the plasmid pRSETA (which allows the translation of the protein binding to the AT2 receptor).

The protein obtained (17 kDa) is purified on a nickel column (Ni-NTA, QuiAexpressionist 07/97, Quiagen), by virtue of the affinity of its six histidine residues for nickel. The protein obtained is then injected into rabbits so as to obtain polyclonal antibodies directed against the ATIP protein. The bleedings obtained have a very good titre.

These antibodies, purified on a GST-ATIP column, after passing through a GST column alone (so as to remove possible GST-specific antibodies and to retain on the GST-ATIP column only the antibodies specific for ATIPmouse-short) may be used successfully to immunoprecipitate and reveal in immunoblotting MYC-ATIPmouse-short from transiently transfected COS cells. Furthermore, this purified antibody also reveals in immunoblotting the ATIPmouse-long protein contained in lysates of COS cells transiently transfected with the plasmid pCDNA3-ATIPmouse-long.

The transfected ATIPmouse-long protein is visualized after SDS-PAGE and immunoblotting with an anti-ATIP antibody, in the form of two polypeptides having apparent molecular weights of 50 and 45 kDa.

This purified antibody was used in immunofluorescence on CHO-hAT2 cells, fixed by a 15-minute treatment with paraformaldehyde (3%). After fixing, the cells are successively treated with solutions of PBS/glycine 50 mM for 20 minutes, PBS/Triton X100 0.1% for 5 minutes and PBS/BSA 0.2% for 15 minutes. They are then successively incubated in solutions containing 15 µg/ml of antibody containing the purified anti-ATIP antibody, and then the anti-rabbit immunoglobulin antibody coupled to rhodamine for 30 minutes. Between each new incubation, three rinses in PBS are carried out. Observations under a fluorescence microscope indicate an expression of the endogenous ATIP protein in the nucleus (predominantly) and in the cytoplasm of the CHO-hAT2 cells.

Some cells show a homogeneous distribution of the fluorescence due to the anti-ATIP antibody in these compartments, whereas other cells which appear more spread out, show a heterogeneous distribution of the fluorescence along the filaments which appear to start from the nucleus and spread up to the plasma membrane of the cell, in an organized network. Additional colocalization experiments should be carried out to determine if these filaments coincide or otherwise with known structures of the cytoskeleton.

EXAMPLE 6: Confirmation of the *in vitro* interaction of the ATIPmouse-short protein with the C-terminal end of the AT2 receptor

To demonstrate the interaction of the ATIPmouse-short protein with the C-terminal end of the AT2 receptor in a system other than that of the two-hybrid system, a protocol which makes it possible to demonstrate this interaction *in vitro* was set up. For that, the fusion protein GST-ATIP as described above

was produced; it is combined through its GST part with glutathione coupled to agarose beads (GA). In parallel, bacteria (DH5α) are transfected with a plasmid (pMAL-c2-AT2, derived from pMAL-c2 from New England Biolabs) encoding a fusion protein between the C-terminal end of the human AT2 receptor (Asn314-Ser363) and MBP (Maltose Binding Protein). These bacteria were cultured and the fusion protein was induced in 0.3 mM IPTG according to the protocol "*pMAL Protein Fusion and Purification System*" from New England Biolabs. After centrifugation of the culture at 4 000 g and solubilization of the pellet obtained in "*column buffer*" (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA), another centrifugation at 9 000 g made it possible to recover a supernatant containing a high concentration of MBP-AT2. This supernatant was brought into contact, for 3 hours at 4°C, with glutathione agarose beads coupled to GST protein alone after addition of NaCl so as to have 300 mM final NaCl. This preincubation step makes it possible to remove the nonspecific interactions which may exist between ATIP and GA-GST. The supernatant recovered was brought into contact with the GA-GST-ATIPmouse-short or GA-GSTalone beads overnight at 4°C. After contact, the beads were rinsed three times in 20 mM Tris-HCl buffer, 300 mM NaCl, 1mM EDTA and once in "column buffer". After analysing the beads rinsed in SDS-PAGE and immunoblotting with an anti-MBP antibody (New England Biolabs), a specific retention of the MBP-AT2 protein is observed on GA-GST-ATIPmouse-short beads which is not observed on the GA-GSTalone beads (Figure 10).

This same protocol was carried out with a plasmid expressing MBP-AT1 (C-terminal end of human AT1 receptor (Leu 297-Glu 359)); it indicates that the MBP-AT1 protein is not retained in a specific manner on the GA-GST-ATIPmouse-short beads (Figure 10).

These results confirm those obtained in the two-hybrid system indicating a specific and selective interaction between the protein according to the

invention and the C-terminal end of the AT2 receptor (and not AT1).

EXAMPLE 7: Modification of the transduction of the signal for the AT2 receptor in clones overexpressing the ATIPmouse-long protein

To verify that the ATIP protein interacts *in vivo* with the AT2 receptor, it was evaluated whether an overexpression of this protein modifies a signal induced by the AT2 receptor.

For that, a stable clone of CHO-hAT2 cells expressing the ATIPmouse-long protein (CHO-hAT2-ATIP), obtained according to the methodology described in Example 4, was used; the functional test for the activity of the AT2 receptor developed on the CHO-hAT2 clone which consists in inhibiting the phosphorylation of the IRβ subunit of the insulin receptor induced by its ligand, was reproduced.

Demonstration of an inhibition by the AT2 receptor of the phosphorylation of IRβ induced by insulin in CHO-hAT2 cells:

The CHO-hAT2 cells are inoculated at a density of 3×10^6 cells per dish having a diameter of 15 cm^2. They are made quiescent by 16 hours of deprivation before being treated. The treatment consists in bringing into contact for 5 minutes with 15 ml of F12 medium containing insulin supplemented or otherwise with CGP42112 (selective agonist of the AT2 receptor). After treatment, the cells are solubilized in lysis buffer containing: 50 mM Hepes, pH 7.6, 1% Triton X-100, 20 mM EDTA, 30 mM sodium pyrophosphate, 30 mM sodium fluoride, 2 mM benzamidine, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride and 1 µg/ml of aprotinin, pepstatin, antipain and leupeptin. The lysates are then subjected to purification on a wheatgerm lectin column, according to the protocol described in Issad, T. et al., (*Eur. J. Biochem.* 1995, **234**, 108-115). After bringing into contact and washings, the lectin beads coupled to

Sepharose (Pharmacia) are recovered in sample buffer containing SDS and the eluted proteins are analysed in SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibodies (Upstate Biotechnology, Inc.) or anti-IRβ antibodies (described in Issad, T. et al., cited above).

The β subunit of the insulin receptor appears as a polypeptide of 97 kDa whose phosphorylation (visualized by revealing with an anti-phosphotyrosine antibody) increases in a dose-dependent manner with the concentration of insulin. Angiotensin II (100 nM) as well as CGP42112 (100 nM) inhibit this phosphorylation at all the insulin doses tested between 0.1 and 0.001 µg/ml (Figure 11). By way of example, CGP42112 inhibits the phosphorylation of IRβ induced by 0.01 µg/ml by a factor of 64 ± 4% (n=7). This result demonstrates that the AT2 receptor interferes negatively with the signalling pathways for the insulin receptor at the initial stage of its activation, which is its autophosphorylation. These results also provide the first evidence of an interconnection between the signalling pathways for the tyrosine kinase receptors and the receptor with seven transmembrane domains which is AT2.

Reproduction of this methodology on CHO-hAT2-ATIP cells:

When this protocol is carried out on CHO-hAT2-ATIP cells, the inhibition by CGP42112 (100 nM) of the phosphorylation of the insulin receptor obtained for various doses of insulin (0.05, 0.01, 0.005, 0.001 µg/ml) is not observed (Figure 11). This result was reproduced 3 times for each of the insulin doses taking, as positive control in each experiment, the inhibition obtained for the clone CHO-hAT2.

This therefore demonstrates that the overexpression of the ATIP protein in the CHO-hAT2 cells interferes with the AT2 receptor signalling,

which confirms the interaction *in vivo* of the ATIP protein with the AT2 receptor.

Another glycosylated protein, retained on a lectin column, having an apparent weight of 120 kDa, identified as being the newly cloned protein SIRP (Kharitonenkov, A. et al., Nature, 1997, 386, 181-186) is phosphorylated on tyrosine in response to insulin. The phosphorylation of this protein, as well as that of IRβ is inhibited in the presence of CGP42112 in the case of the clone CHO-hAT2 and is not in the case of the clone CHO-hAT2-ATIP. This confirms that the ATIP protein interferes with the signalling pathways for the AT2 receptor. These results indeed show the possible value of the use of the ATIP protein for modifying signalling mediated by the AT2 receptor and for possibly compensating for pathological conditions associated with abnormalities in the regulation of this receptor.

As is evident from the above, the invention is not at all limited to those of its embodiments, implementations and applications which have just been described more explicitly; it encompasses, on the contrary, all the variants thereof which may occur to the specialist in the field, without departing from the framework or the scope of the present invention.